

A hydrolase enzyme inactivating endogenous ligands for cannabinoid receptors

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Abstract : Cannabinoids are psychoactive components of marijuana, and bind to specific G protein-coupled receptors in the brain and other mammalian tissues. Anandamide (arachidonoyl ethanolamide) was discovered as an endogenous agonist for the cannabinoid receptors. Hydrolysis of anandamide to arachidonic acid and ethanolamine results in the loss of its biological activities. The enzyme responsible for this hydrolysis was solubilized, partially purified from the microsomes of porcine brain, and referred to as anandamide amidohydrolase. In addition to the anandamide hydrolysis, the enzyme preparation catalyzed anandamide synthesis by the condensation of arachidonic acid with ethanolamine. Several lines of enzymological evidence suggested that a single enzyme catalyzes both the hydrolysis and synthesis of anandamide. This reversibility was confirmed by the use of a recombinant enzyme of rat liver overexpressed in COS-7 cells. However, in consideration of the high K_m value for ethanolamine as a substrate for the anandamide synthesis, the enzyme was presumed to act as a hydrolase rather than a synthase under physiological conditions. The recombinant enzyme acted not only as an amidase hydrolyzing anandamide and other fatty acid amides but also as an esterase hydrolyzing methyl ester of arachidonic acid. 2-Arachidonoylglycerol, which was found recently to be another endogenous ligand, was also efficiently hydrolyzed by the esterase activity of the same enzyme. The anandamide hydrolase and synthase activities were detected in a variety of rat organs, and liver showed by far the highest activities. A high anandamide hydrolase activity was also detected in small intestine but only after the homogenate was precipitated with acetone to remove endogenous lipids inhibiting the enzyme activity. The distribution of mRNA of the enzyme was in agreement with that of the enzyme activity. *J. Med. Invest.* 45 : 27-36, 1998

Key words : cannabinoid, anandamide, 2-arachidonoylglycerol, arachidonic acid, amidohydrolase

INTRODUCTION

Marijuana, prepared from the plant *Cannabis sativa* L., has long been used for therapeutic and recreational purposes (1). A major psychoactive component in marijuana was isolated and identified as Δ^9 -tetrahydrocannabinol (Fig.1a) in 1964 (2). Its structurally related compounds are collectively

referred to as cannabinoids. Synthetic compounds with more potent cannabimimetic activities were later developed, leading to the discovery of a seven-transmembrane G protein-coupled receptor specific for cannabinoids in the brain (3). A cDNA for this cannabinoid receptor was then cloned from rat brain in 1990 (4), and another isoform of the receptor from human leukemia HL-60 cells in 1993 (5). The former receptor is now referred to as CB1 and the latter as CB2 (5). CB1 is expressed mainly in the brain and to a lesser extent in various peripheral tissues while CB2 is expressed mostly in immune systems like tonsil, spleen, macrophages

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and lymphocytes (6). When cannabinoids bind to the receptors, they inhibit forskolin-stimulated adenylyl cyclase activity and N- and P/Q-type calcium channels through pertussis toxin-sensitive G protein (7). Agonists and antagonists specific for either CB1 or CB2 are now being developed for a variety of clinical applications (8). Particularly, CB2-specific agonists are expected to be effective as anti-inflammatory, immunosuppressive and analgesic agents and active in the treatment of glaucoma and chemotherapy-induced emesis.

In 1992 an endogenous agonist for the receptor was isolated from porcine brain, and its chemical structure was determined as arachidonylethanolamide (Fig.1b) (9). The compound was termed as anandamide, which was derived from "ananda" meaning bliss in Sanskrit (9). Anandamide was reported to show cannabimimetic activities in various pharmacological and behavioral experiments (for review, 10-12). Later, 2-arachidonoylglycerol was also found to be an endogenous ligand for the cannabinoid receptors (Fig.1c) (13, 14).

Anandamide loses its biological activities when it is enzymatically hydrolyzed to arachidonic acid and ethanolamine (Fig.2a). The enzyme responsible for this hydrolysis is referred to as anandamide amidase (15), anandamide amidohydrolase (16) or fatty-acid amide hydrolase (17). In this article we will discuss recent progress in the research on this enzyme including our experimental results.

PARTIAL PURIFICATION AND CHARACTERIZATION OF ANANDAMIDE AMIDOHYDROLASE

In 1993 Deutsch and Chin first reported the enzymatic hydrolysis of anandamide by the membrane fractions of several rat tissues and cells, especially brain (15). Later other groups also reported this enzyme activity in various mammalian tissues and cell lines (16, 18-22). We were interested in the metabolism of anandamide in porcine brain from which anandamide was first isolated (9).

When the homogenate of porcine brain was incubated with [arachidonoyl-1-¹⁴C]anandamide, the production of [1-¹⁴C]arachidonic acid was observed as analyzed by thin-layer chromatography (TLC) (23). Sequential centrifugation revealed the highest enzyme activity in the 105,000 x g pellet (microsomal fraction), and its specific activity was about 7.5 nmol/min/mg protein at 37°C. The enzyme could be solubilized from the microsomes with 1% Triton X-100. In order to purify the enzyme we attempted several column chromatographies. However, probably due to hydrophobicity of the enzyme protein, yield of the enzyme activity was low in most cases. The preparation with the highest specific activity was obtained by hydrophobic chromatography with a Tosoh Phenyl-5PW column. By this method anandamide amidohydrolase was purified 22-fold (a specific enzyme activity, 0.37 μ mol/min/mg protein, Peak II in Fig.3) from the solubilized enzyme prepa-

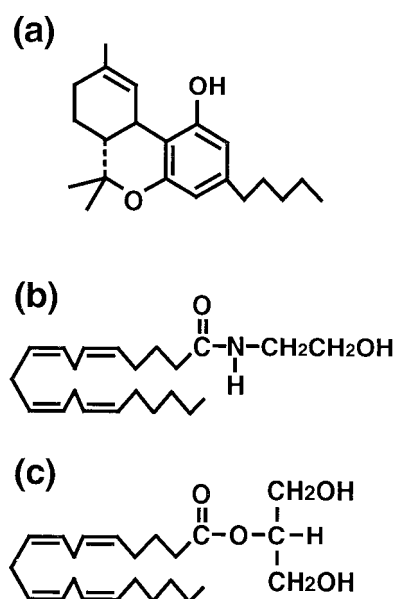


Fig.1. Cannabinoid receptor agonists. (a) Δ^9 -tetrahydrocannabinol, (b) anandamide and (c) 2-arachidonoylglycerol.

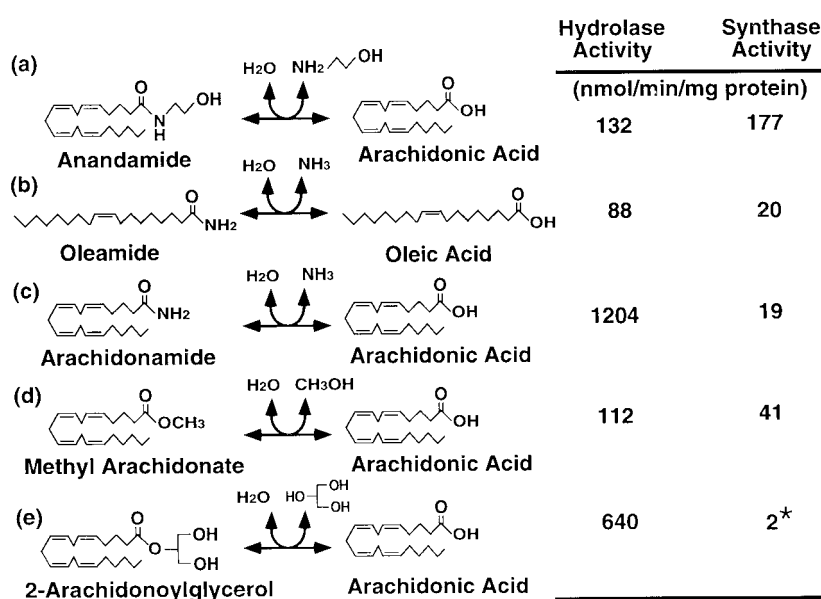


Fig.2. Reversible reactions catalyzed by anandamide amidohydrolase. Hydrolase and synthase activities for the substrates as indicated were determined by the use of the particulate fractions of COS-7 cells overexpressing anandamide amidohydrolase of rat liver (32, 35). *Products were not identified.

ration.

According to previous reports (15, 24, 25), the enzyme preparation of brain catalyzed not only the anandamide hydrolysis but also its reverse reaction, namely, the anandamide synthesis by the condensation of arachidonic acid and ethanolamine (Fig.2 a). When we allowed the Triton X-100-solubilized proteins of porcine brain microsomes to react with [14 C]arachidonic acid in the presence of ethanolamine, [14 C]anandamide was formed. This "anandamide synthase" was co-purified to a specific enzyme activity of 0.16 μ mol/min/mg protein along with the anandamide amidohydrolase by the Phenyl-5PW column (Peak II in Fig.3). The hydrolase and synthase were also co-eluted from a Tosoh DEAE-5PW column.

The hydrolase and synthase reactions catalyzed by the partially purified enzyme proceeded linearly up to 20 min, and the activities were dependent on the amount of the enzyme protein. Optimal pH was 7.5-9.0 for both the reactions. As compared at 300 μ M, the enzyme hydrolyzed ethanolamides of linoleic acid, oleic acid and palmitic acid at 44%, 27% and 19% the rate of the anandamide hydrolysis. Such a wide specificity for fatty acid species suggests that anandamide amidohydrolase is identical to *N*-acylethanolamine amidohydrolase, which was earlier shown to be capable of reacting with various fatty acid ethanolamides (26). When the synthase activity was examined in the presence of ethanolamine, the enzyme converted arachidonic, linoleic, oleic, and palmitic acids to their ethanolamides, and the reaction rates differed little among these fatty acids.

We tested inhibitory effects of several compounds on the hydrolase and synthase activities (Fig.4). Arachidonyl trifluoromethyl ketone (ATFMK),

which was reported originally as an inhibitor for cytosolic phospholipase A_2 and later as that for anandamide amidohydrolase (27), inhibited the hydrolase and synthase activities in parallel with IC_{50} values of about 1 μ M. *p*-Chloromercuribenzoic acid (PCMB), a sulfhydryl-reactive agent, and phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP), serine hydrolase inhibitors, also inhibited both the activities almost in parallel. All these results suggest that the anandamide hydrolase and synthase

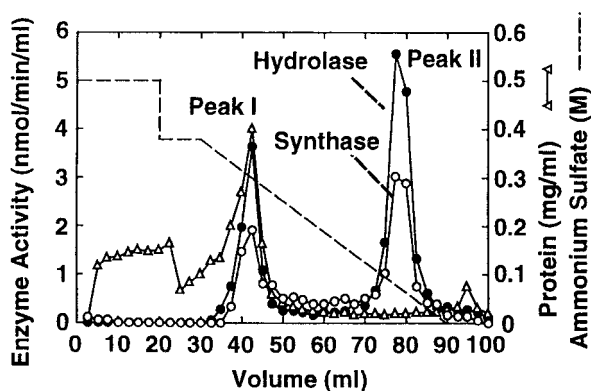


Fig.3. Partial purification of anandamide amidohydrolase from the microsomes of porcine brain by hydrophobic chromatography. Triton X-100-solubilized proteins of the porcine brain microsomes were applied to a Phenyl-5PW column. Each fraction was assayed for the anandamide hydrolase and synthase activities.

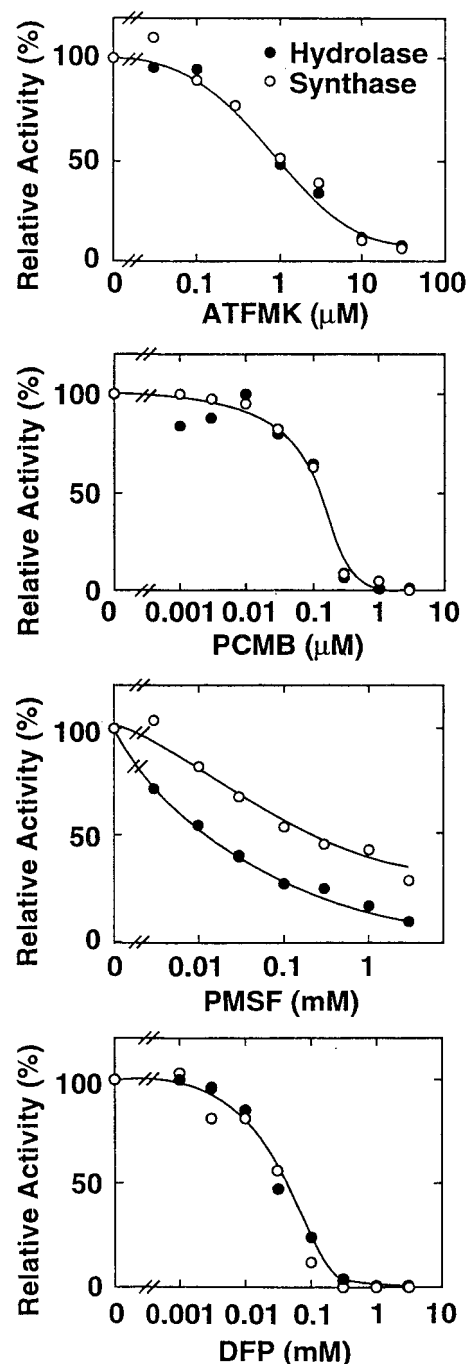


Fig.4. Inhibition of the anandamide hydrolase and synthase activities by various compounds. The enzyme partially purified from porcine brain was used.

activities are derived from a single enzyme protein (23).

We considered the possibility that the anandamide hydrolase activity was attributable to other known amidohydrolases. A possible involvement of ceramidase, which is an enzyme hydrolyzing ceramide to sphingosine and fatty acid, was ruled out since the partially purified enzyme was essentially inactive with [14 C]ceramide (*N*-oleoylsphingosine) as a substrate. In addition, several artificial hydrophobic substrates for proteases (peptidyl 4-methyl-coumaryl-7-amide substrates obtained from Peptide Institute, Osaka) were inactive with the enzyme preparation (23).

CHARACTERIZATION OF RECOMBINANT ANANDAMIDE AMIDOHYDROLASE

Primary amide of oleic acid (oleamide) (Fig.2b) was identified as an endogenous sleep inducer, and was shown to be hydrolyzed enzymatically (28). In 1996 Cravatt *et al.* cloned a cDNA for an enzyme hydrolyzing oleamide from a rat liver cDNA library (17). The enzyme was composed of 579 amino acids, and its molecular weight was calculated as 63 kDa. Later, human and mouse homologues of this enzyme were also cloned by the same group (29). They found that the recombinant enzyme expressed in COS-7 cells hydrolyzed not only oleamide but also anandamide, and referred to the enzyme as fatty-acid amide hydrolase (17). Maurelli *et al.* reported that an anandamide amidohydrolase of N18 mouse neuroblastoma cells was active with oleamide (30), and we also showed that the partially purified porcine enzyme could hydrolyze oleamide as well as anandamide (31). Thus, fatty-acid amide hydrolase seems to be identical to anandamide amidohydrolase.

Based on the reported cDNA sequence, we prepared a cDNA for the coding region of fatty-acid amide hydrolase by reverse-transcriptase polymerase chain reaction using rat liver mRNA as a template (32). The cDNA was inserted to an eukaryotic expression vector pcDNA 3.1(+) (Invitrogen), and COS-7 cells were transfected with the recombinant vector. After 3 days the cells were harvested, sonicated, and centrifuged at 267,000 \times g. The resultant pellet (particulate fraction) was used as the recombinant enzyme preparation in the following experiments. When the enzyme was allowed to react with [14 C]anandamide, radioactive arachidonic acid was produced depending on the concentrations of anandamide (Fig.5a). The specific enzyme activity was 132 nmol/min/mg protein

at 37°C, and the K_m value for anandamide was about 20 μ M. The particulate fraction of the control cells, transfected with the insert-free vector, did not show the hydrolase activity. The enzyme also converted [14 C]arachidonic acid to [14 C]anandamide in the presence of ethanolamine. This activity depended on the concentrations of arachidonic acid and ethanolamine (Figs.5b and 5c), and the K_m values for arachidonic acid and ethanolamine were 190 μ M and 36 mM, respectively. The specific synthase activity was 177 nmol/min/mg protein. The control cells did not

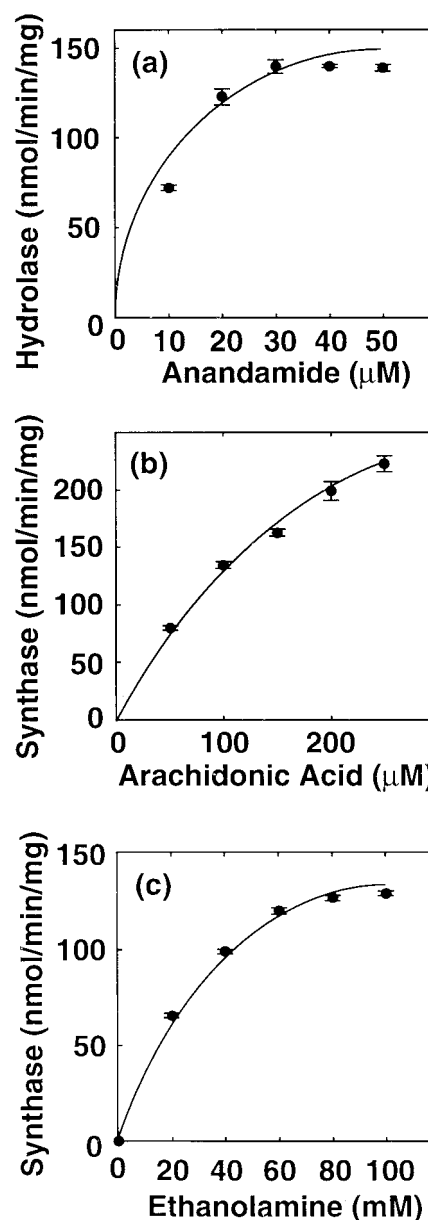


Fig. 5. Dependency of the anandamide hydrolase and synthase activities on the substrate concentration. The particulate fraction of COS-7 cells overexpressing anandamide amidohydrolase of rat liver was allowed to react (a) with different concentrations of [14 C]anandamide or (b) with different concentrations of [14 C]arachidonic acid in the presence of 250 mM ethanolamine or (c) with different concentrations of ethanolamine in the presence of 200 μ M [14 C]arachidonic acid.

synthesize anandamide. These results confirmed the reversibility of the enzymatic anandamide hydrolysis. Deutsch's group also showed the reversibility of this reaction with the recombinant enzyme (33). However, due to the extremely high K_m value for ethanolamine, the enzyme appears to function as the hydrolase rather than the synthase under physiological conditions.

We also examined the reactivity of the recombinant enzyme with other fatty acid derivatives (32). Oleamide was hydrolyzed by the recombinant enzyme as reported by Cravatt *et al.* (17) (Fig.2b). We found that the same enzyme preparation could form oleamide from oleic acid in the presence of as high as 1 M ammonium chloride. In agreement with this finding, a recent report showed the oleamide synthesis through this pathway by brain microsomes (34). Arachidonamide was also hydrolyzed by the same enzyme, and in its reverse reaction this compound was formed from arachidonic acid and ammonium chloride (Fig.2c). Furthermore, methyl ester of arachidonic acid (Fig.2d) was hydrolyzed by this enzyme, but not by the control cells. In the reverse reaction, arachidonic acid was converted to its methyl ester in the presence of 2 M methanol. It should be noted that this ester formation preferred acidic pH around 5 although alkaline pH was optimum for the amide synthesis. As compared between the substrates at 100 μ M, the hydrolase activity was by far the most active with arachidonamide (Fig.2). Oleamide and methyl arachidonate were as active as anandamide. For the synthase reaction with 200 μ M fatty acid as a substrate, the anandamide synthesis proceeded much faster than the syntheses of primary amides and ester (Fig.2).

Considering that anandamide amidohydrolase acted as an esterase for methyl arachidonate, we were interested to know whether or not the enzyme hydrolyzed 2-arachidonoylglycerol, another endogenous ligand for the cannabinoid receptor (Fig.2e) (35). The recombinant enzyme was allowed to react with 2-arachidonoylglycerol for 1 min at 37°C. When the ethereal extract was analyzed by reverse-phase high-performance liquid chromatography monitoring absorption at 205 nm, arachidonic acid was detected. In contrast, this hydrolytic reaction did not occur with the control cells. The enzymatic hydrolysis of 2-[¹⁴C]arachidonoylglycerol was also observed by TLC. The hydrolysis of 2-arachidonoylglycerol proceeded about 4-fold faster than the anandamide hydrolysis with a K_m value as low as 6 μ M and an optimal pH of 10. PMSF and methyl arachidonyl

fluorophosphonate (MAFP) inhibited the enzymatic hydrolysis of both anandamide and 2-arachidonoylglycerol in parallel. Furthermore, the hydrolysis of radioactive 2-arachidonoylglycerol and anandamide was competitively inhibited by non-radioactive anandamide and 2-arachidonoylglycerol, respectively. 1(3)-Arachidonoylglycerol was as active as 2-arachidonoylglycerol, but 1(3)-oleoylglycerol was less active. In contrast to monoacylglycerol, diacylglycerols such as 1-stearoyl-2-arachidonoylglycerol were totally inactive. Although monoacylglycerol lipase and other esterases were reported to hydrolyze monoacylglycerols including 2-arachidonoylglycerol (36), our results suggest that anandamide and 2-arachidonoylglycerol can be inactivated by the same enzyme. Recently Di Marzo *et al.* reported that anandamide amidohydrolase partially purified from mouse neuroblastoma cells N18TG2 and rat basophilic leukemia cells RBL-2H3 could hydrolyze 2-arachidonoylglycerol (37).

DISTRIBUTION OF ANANDAMIDE AMIDOHYDROLASE IN ANIMAL ORGANS

In order to examine the organ distribution of anandamide amidohydrolase in rats, we screened both the anandamide hydrolase and synthase activities in homogenates of various rat organs (38). As presented in Fig.6a, liver showed by far the highest specific activities of the hydrolase and synthase (4-5 nmol/min/mg protein). Considerable activities were also detected in cerebrum, cerebellum, testis and parotid gland. In most of the organs tested, the synthase activity was comparable to the hydrolase activity. However, in the case of small intestine, the synthase activity was much higher than the hydrolase activity. Since the homogenate of small intestine inhibited the hydrolase activity of rat liver microsomes, we presumed the presence of endogenous inhibitory factors in the small intestine homogenate. The factors were heat-stable and extractable with acetone. In order to identify the inhibitory factors, we subjected the acetone extract of the homogenate to TLC, and scraped various lipid bands from silica gel. Then, we eluted the lipids from silica gel with methanol, and tested for their inhibitory effects. The bands corresponding to free fatty acids, monoacylglycerols and polar lipids inhibited the hydrolase and synthase activities. In agreement with this result, when pure free oleic acid, 2-arachidonoylglycerol or phosphatidylcholine was included in the reaction

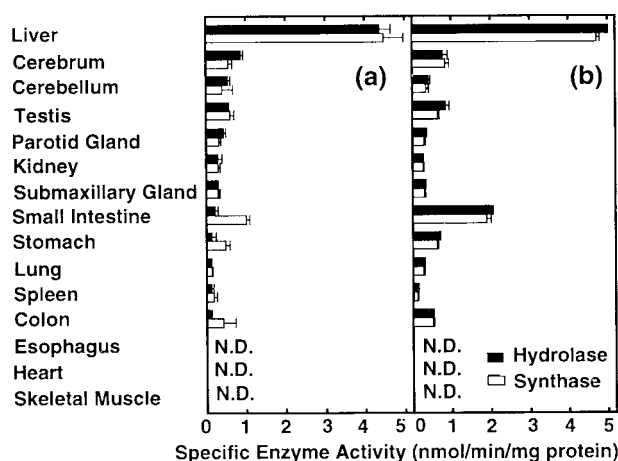


Fig. 6. Organ distribution of anandamide amidohydrolase in rats. (a) Homogenates prepared from various rat organs were assayed for the anandamide hydrolase and synthase activities. (b) The homogenates were treated with 90% cold acetone. The precipitated proteins were subjected to the enzyme assays. N.D., below detection limit.

mixture, the anandamide hydrolase and synthase activities of the liver microsome were reduced depending on the concentrations of these lipids. It should be noted that the hydrolase activity was inhibited more potently than the synthase activity.

In consideration of such endogenous lipid inhibitors, we treated the homogenate of small intestine with 90% cold acetone, and the resultant pellet was assayed for the enzyme activities. As we expected, the specific hydrolase activity of the homogenate was increased about 10-fold by the acetone precipitation. We then reexamined the organ distribution of the hydrolase and synthase activities with the acetone-treated homogenates. As shown in Fig. 6b, small intestine had a high specific activity of the hydrolase (2.0 nmol/min/mg protein), second only to liver. Stomach and colon also showed considerable activities of the hydrolase. Furthermore, we examined distribution of the anandamide amidohydrolase mRNA in rat organs. As analyzed by Northern blotting using 25 µg of total RNA prepared from various organs, an intense band around 2.5 kb was detected in small intestine and stomach as well as liver. Faint bands at the same position were observed with brain, testis, parotid gland, kidney, submaxillary gland and spleen.

Previously, Desarnaud *et al.* reported that the hydrolase activity of rat was high in liver, brain and testis, and low in small intestine (16). Our results demonstrated considerable activities of anandamide amidohydrolase in the gastrointestinal tract like small intestine, stomach and colon. The enzyme in gastrointestinal tract may play a role in detoxifying

exogenous bioactive fatty acid amides such as anandamide and oleamide. Alternatively, small intestine is a target organ of cannabinoid as shown by the experimental result that electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation are inhibited by various cannabinoids (39). Therefore, the enzyme may be involved in the regulation of anandamide concentration in the small intestine.

Since anandamide reduced intraocular pressure and caused conjunctival hyperemia (40, 41), we investigated the distribution of anandamide amidohydrolase in ocular tissues. When the homogenates of various tissues of porcine eyes were assayed, retina, choroid, iris, optic nerve and lacrimal gland showed high specific activities of the anandamide hydrolase and synthase comparable to those in porcine brain (42). When the subcellular distribution of the enzyme in retina was examined, the enzyme activity was mostly recovered in the particulate fractions rather than in the cytosol in agreement with its distribution in the brain. Lens did not show either the hydrolase activity or the synthase activity.

INHIBITORS FOR ANANDAMIDE AMIDO-HYDROLASE

Since anandamide amidohydrolase is so far known as the only enzyme capable of hydrolyzing anandamide, the enzyme is thought to play an important role in regulating biological activities of anandamide *in vivo*. In fact, PMSF capable of inhibiting the enzyme potentiates the apparent affinity of anandamide for cannabinoid receptors (43), and anandamide derivatives resistant to the enzymatic hydrolysis like (*R*)-(+)-arachidonyl-1'-hydroxy-2'-propylamide (termed as *R*-metanandamide) show more potent cannabimimetic activities than anandamide (44). Therefore, potent specific inhibitors for anandamide amidohydrolase may be useful tools to elucidate the physiological and pathophysiological significance of anandamide. As discussed above, generally used serine hydrolase inhibitors such as PMSF and DFP and sulfhydryl group blockers like PCMB were reported to inhibit anandamide amidohydrolase (15, 23, 26). Later, several fatty acid derivatives such as ATFMK (27), MAFP (45, 46), palmitylsulfonyl fluoride (47) and arachidonoyl-diazo-methyl-ketone (ADMK) (46) were reported to inhibit anandamide amidohydrolase (Fig. 7). MAFP is the most potent irreversible inhibitor so far reported with an IC_{50}

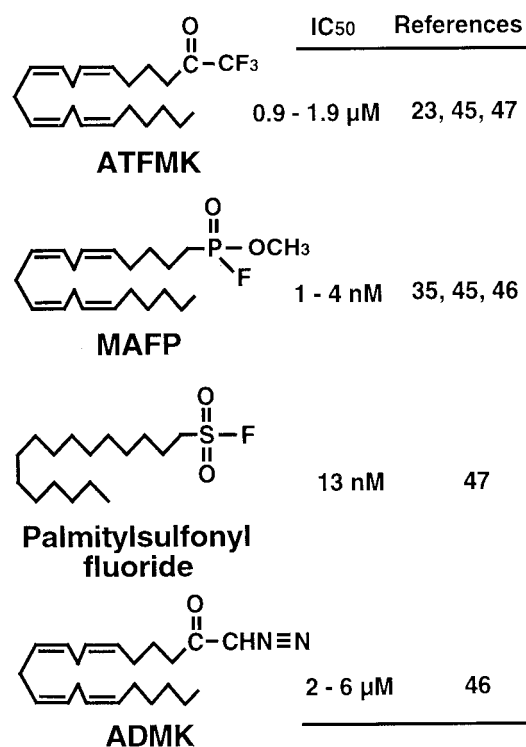


Fig.7. Representative fatty acid derivatives inhibiting anandamide amidohydrolase. Reported IC₅₀ values for the anandamide hydrolysis are shown.

value of as low as 1-4 nM. We confirmed the inhibitory effects of ATFMK (23), MAFP (35, 46) and ADMK (46) by the use of the partially purified porcine enzyme or the recombinant rat enzyme. However, most of these fatty acid derivatives were originally developed as phospholipase A₂ inhibitors, and are not strictly specific for anandamide amidohydrolase. The development of more selective inhibitors is awaited. The inhibitors may be useful for the treatment of glaucoma or as analgesic, anti-emetic, immunosuppressive agents and appetite enhancers by increasing the endogenous level of anandamide (8).

OVERALL METABOLISM OF ANANDAMIDE

We summarize here the currently accepted metabolic pathway of anandamide. As mentioned above, anandamide amidohydrolase is an enzyme catalyzing a reversible reaction. However, its catalytic properties suggest that the enzyme is responsible for the hydrolysis of anandamide under physiological conditions. The most likely biosynthetic pathway of anandamide is as follows (Fig.8) (48-50). The first step is the arachidonyl transfer from glycerophospholipids

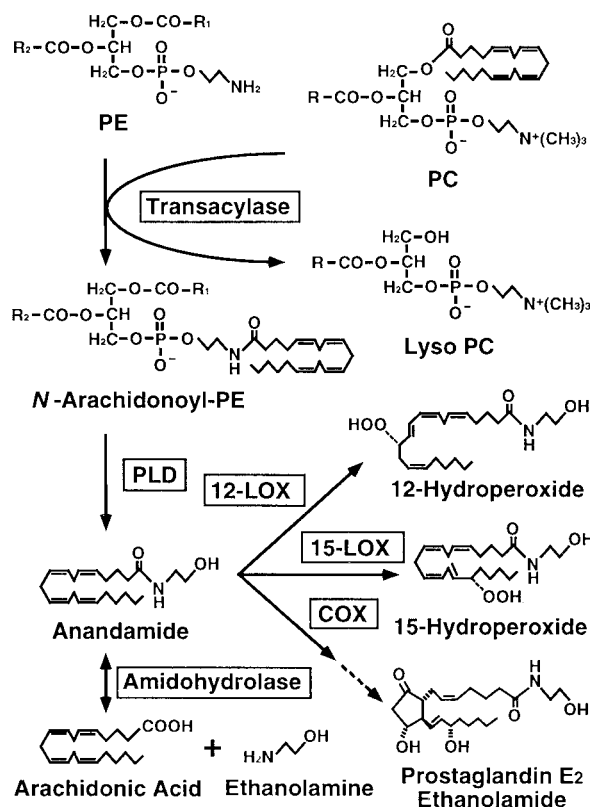


Fig.8. Metabolic pathways of anandamide. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PLD, phospholipase D; LOX, lipoxygenase; COX, cyclooxygenase.

like phosphatidylcholine to phosphatidylethanolamine catalyzed by a calcium-dependent transacylase. The produced *N*-arachidonoyl-phosphatidylethanolamine is cleaved by a phospholipase D, and anandamide is released. It should be noted that *N*-acylethanolamines containing various fatty acid species are produced together with anandamide through this pathway. Details on the enzymes involved in this synthetic route are still unclear. Anandamide thus formed is quickly hydrolyzed to arachidonic acid and ethanolamine by anandamide amidohydrolase widely distributed in the animal tissues. In addition, we (51) and others (52) showed that the arachidonate moiety of anandamide is oxygenated by 12- and 15-lipoxygenases (Fig.8). Oxygenation of anandamide by cytochrome P450 (53) and cyclooxygenase (prostaglandin endoperoxide synthase) (54) was also reported.

REFERENCES

1. Mechoulam R: The pharmacohistory of *Cannabis Sativa*. In: Mechoulam R, ed. *Cannabinoids as Therapeutic Agents*. CRC Press, Boca Raton,

- 1986, pp.1-19
2. Gaoni Y, Mechoulam R: Isolation, structure, and partial synthesis of an active constituent of Hashish. *J Am Chem Soc* 86 : 1646-1647, 1964
3. Devane WA, Dysarz FA III, Johnson MR, Melvin LS, Howlett AC : Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* 34 : 605-613, 1988
4. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI : Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346 : 561-564, 1990
5. Munro S, Thomas KL, Abu-Shaar M : Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365 : 61-65, 1993
6. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur, G, Casellas P : Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem* 232 : 54-61, 1995
7. Pertwee RG : Pharmacology of cannabinoid CB₁ and CB₂ receptors. *Pharmacol Ther* 74 : 129-180, 1997
8. Barth F : Cannabinoid receptor agonists and antagonists. *Exp Opin Ther Patents* 8 : 301-313, 1998
9. Devane WA, Hanuš L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, Mechoulam R : Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258 : 1946-1949, 1992
10. Mechoulam R, Hanuš L, Martin BR : Search for endogenous ligands of the cannabinoid receptor. *Biochem Pharmacol* 48 : 1537-1544, 1994
11. Di Marzo V : 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties : biochemistry and possible physiopathological relevance. *Biochim Biophys Acta* 1392 : 153-175, 1998
12. Felder CC, Glass M : Cannabinoid receptors and their endogenous agonists. *Annu Rev Pharmacol Toxicol* 38 : 179-200, 1998
13. Mechoulam R, Ben-Shabat S, Hanuš L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, Compton DR, Pertwee RG, Griffin G, Bayewitch M, Barg J, Vogel Z : Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* 50 : 83-90, 1995
14. Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A, Waku K : 2-Arachidonoylglycerol : a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* 215 : 89-97, 1995
15. Deutsch DG, Chin SA : Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* 46 : 791-796, 1993
16. Desarnaud F, Cadas H, Piomelli D : Anandamide amidohydrolase activity in rat brain microsomes Identification and partial characterization. *J Biol Chem* 270 : 6030-6035, 1995
17. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB : Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384 : 83-87, 1996
18. Di Marzo V, Fontana A, Cadas H., Schinelli S., Cimino G, Schwartz J-C, Piomelli D : Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* 372 : 686-691, 1994
19. Hillard CJ, Wilkison DM, Edgemond WS, Campbell WB : Characterization of the kinetics and distribution of *N*-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim Biophys Acta* 1257 : 249-256, 1995
20. Watanabe K, Kayano Y, Matsunaga T, Yamamoto I, Yoshimura H : Inhibition of anandamide amidase activity in mouse brain microsomes by cannabinoids. *Biol Pharm Bull* 19 : 1109-1111, 1996
21. Paria BC, Deutsch DG, Dey SK : The uterus is a potential site for anandamide synthesis and hydrolysis : differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. *Mol Reprod Dev* 45 : 183-192, 1996
22. Bisogno T, Maurelli S, Melck D, De Petrocellis L, Di Marzo V : Biosynthesis, uptake, and degradation of anandamide and palmitoylethanolamide in leukocytes. *J Biol Chem* 272 : 3315-3323, 1997
23. Ueda N, Kurahashi Y, Yamamoto S, Tokunaga T : Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem* 270 : 23823-23827, 1995
24. Kruszka KK, Gross RW : The ATP- and CoA-independent synthesis of arachidonylethanolamide. *J Biol Chem* 269 : 14345-14348, 1994
25. Devane WA, Axelrod J : Enzymatic synthesis of anandamide, an endogenous ligand for the cannabinoid receptor, by brain membranes.

- Proc Natl Acad Sci USA 91 : 6698-6701, 1994
26. Schmid PC, Zuzarte-Augustin ML, Schmid HHO : Properties of rat liver *N*-acylethanolamine amidohydrolase. J Biol Chem 260:14145-14149, 1985
 27. Koutek B, Prestwich GD, Howlett AC, Chin SA, Salehani D, Akhavan N, Deutsch DG : Inhibitors of arachidonoyl ethanolamide hydrolysis. J Biol Chem 269 : 22937-22940, 1994
 28. Cravatt BF, Prospero-Garcia O, Siuzdak G, Gilula NB, Henriksen SJ, Boger DL, Lerner RA : Chemical characterization of a family of brain lipids that induce sleep. Science 268 : 1506-1509, 1995
 29. Giang DK, Cravatt BF : Molecular characterization of human and mouse fatty acid amide hydrolases. Proc Natl Acad Sci USA 94 : 2238-2242, 1997
 30. Maurelli S, Bisogno T, De Petrocellis L, Di Luccia A, Marino G, Di Marzo V : Two novel classes of neuroactive fatty acid amides are substrates for mouse neuroblastoma 'anandamide amidohydrolase'. FEBS Lett 377 : 82-86, 1995
 31. Ueda N, Kurahashi Y, Yamamoto K, Yamamoto S : Anandamide amidohydrolase from porcine brain Partial purification and characterization. In : Honn KV, Marnett LJ, Nigam S, Jones RL, Wong P Y-K, eds. Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation, and Radiation Injury 3. Plenum Press, New York, 1997, pp. 323-328
 32. Kurahashi Y, Ueda N, Suzuki H, Suzuki M, Yamamoto S : Reversible hydrolysis and synthesis of anandamide demonstrated by recombinant rat fatty-acid amide hydrolase. Biochem Biophys Res Commun 237 : 512-515, 1997
 33. Arreaza G, Devane WA, Omeir RL, Sajani G, Kunz J, Cravatt BF, Deutsch DG : The cloned rat hydrolytic enzyme responsible for the breakdown of anandamide also catalyzes its formation via the condensation of arachidonic acid and ethanolamine. Neurosci Lett 234 : 59-62, 1997
 34. Sugiura T, Kondo S, Kodaka T, Tonegawa T, Nakane S, Yamashita A, Ishima Y, Waku K : Enzymatic synthesis of oleamide (*cis*-9, 10-octadecenoamide), an endogenous sleep-inducing lipid, by rat brain microsomes. Biochem Mol Biol Int 40 : 931-938, 1996
 35. Goparaju SK, Ueda N, Yamaguchi H, Yamamoto S : Anandamide amidohydrolase reacting with 2-arachidonoylglycerol, another cannabinoid receptor ligand. FEBS Lett 422 : 69-73, 1998
 36. Chau L-Y, Tai H-H : Monoglyceride and diglyceride lipases from human platelet microsomes. Biochim Biophys Acta 963 : 436-444, 1988
 37. Di Marzo V, Bisogno T, Sugiura T, Melck D, De Petrocellis L : The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells : connections with anandamide. Biochem J 331 : 15-19, 1998
 38. Katayama K, Ueda N, Kurahashi Y, Suzuki H, Yamamoto S, Kato I : Distribution of anandamide amidohydrolase in rat tissues with special reference to small intestine. Biochim Biophys Acta 1347 : 212-218, 1997
 39. Pertwee RG, Fernando SR, Nash JE, Coutts AA : Further evidence for the presence of cannabinoid CB₁ receptors in guinea-pig small intestine. Br J Pharmacol 118 : 2199-2205, 1996
 40. Pate DW, Jarvinen K, Urtti A, Jarho P, Jarvinen T : Ophthalmic arachidonylethanolamide decreases intraocular pressure in normotensive rabbits. Curr Eye Res 14 : 791-797, 1995
 41. Mikawa Y, Matsuda S, Kanagawa T, Tajika T, Ueda N, Mimura Y : Ocular activity of topically administered anandamide in the rabbit. Jpn J Ophthalmol 41 : 217-220, 1997
 42. Matsuda S, Kanemitsu N, Nakamura A, Mimura Y, Ueda N, Kurahashi Y, Yamamoto S : Metabolism of anandamide, an endogenous cannabinoid receptor ligand, in porcine ocular tissues, Exp Eye Res 64 : 707-711, 1997
 43. Childers SR, Sexton T, Roy MB : Effects of anandamide on cannabinoid receptors in rat brain membranes. Biochem Pharmacol 47 : 711-715, 1994
 44. Abadji V, Lin S, Taha G, Griffin G, Stevenson LA, Pertwee RG, Makriyannis A : (*R*)-Methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability. J Med Chem 37 : 1889-1893, 1994
 45. Deutsch DG, Omeir R, Arreaza G, Salehani D, Prestwich GD, Huang Z, Howlett A : Methyl arachidonoyl fluorophosphonate : a potent irreversible inhibitor of anandamide amidase. Biochem Pharmacol 53 : 255-260, 1997
 46. De Petrocellis L, Melck D, Ueda N, Maurelli S, Kurahashi Y, Yamamoto S, Marino G, Di Marzo V : Novel inhibitors of brain, neuronal, and basophilic anandamide amidohydrolase. Biochem Biophys Res Commun 231 : 82-88, 1997
 47. Deutsch DG, Lin S, Hill WAG, Morse KL, Salehani

- D, Arreaza G, Omeir RL, Makriyannis A : Fatty acid sulfonyl fluorides inhibit anandamide metabolism and bind to the cannabinoid receptor. *Biochem Biophys Res Commun* 231 : 217-221, 1997
48. Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A, Ishima Y, Waku K: Transacylase-mediated and phosphodiesterase-mediated synthesis of *N*-arachidonylethanolamine, an endogenous cannabinoid-receptor ligand, in rat brain microsomes Comparison with synthesis from free arachidonic acid and ethanolamine. *Eur J Biochem* 240 : 53-62, 1996
49. Cadas H, Gaillet S, Beltramo M, Venance L, Piomelli D : Biosynthesis of an endogenous cannabinoid precursor in neurons and its control by calcium and cAMP. *J Neurosci* 16 : 3934-3942, 1996
50. Schmid HHO, Schmid PC, Natarajan V : The *N*-acylation-phosphodiesterase pathway and cell signalling. *Chem Phys Lipids* 80 : 133-142, 1996
51. Ueda N, Yamamoto K, Yamamoto S, Tokunaga T, Shirakawa E, Shinkai H, Ogawa M, Sato T, Kudo I, Inoue K, Takizawa H, Nagano T, Hirobe M, Matsuki N, Saito H : Lipoxygenase-catalyzed oxygenation of arachidonylethanolamide, a cannabinoid receptor agonist. *Biochim Biophys Acta* 1254 : 127-134, 1995
52. Hampson AJ, Hill WAG, Zan-Phillips M, Makriyannis A, Leung E, Eglen RM, Bornheim LM: Anandamide hydroxylation by brain lipoxygenase : metabolite structures and potencies at the cannabinoid receptor. *Biochim Biophys Acta* 1259 : 173-179, 1995
53. Bornheim LM, Kim KY, Chen B, Correia MA : The effect of cannabidiol on mouse hepatic microsomal cytochrome P450-dependent anandamide metabolism. *Biochem Biophys Res Commun* 197 : 740-746, 1993
54. Yu M, Ives D, Ramesha CS : Synthesis of prostaglandin E₂ ethanolamide from anandamide by cyclooxygenase-2. *J Biol Chem* 272 : 21181-21186, 1997